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E2F Activators Signal and Maintain Centrosome Amplification in Breast Cancer Cells

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Centrosomes ensure accurate chromosome segregation by directing spindle bipolarity. Loss of centrosome regulation results in centrosome amplification, multipolar mitosis and aneuploidy. Since centrosome amplification is common in premalignant lesions and breast tumors, it is proposed to play a central role in breast tumorigenesis, a hypothesis that remains to be tested. The coordination between the cell and centrosome cycles is of paramount importance to maintain normal centrosome numbers, and the E2Fs may be responsible for regulating these cycles. However, the role of E2F activators in centrosome amplification is unclear. Because E2Fs are deregulated in Her2$^+$ cells displaying centrosome amplification, we addressed whether they signal this abnormal process. Knockdown of E2F1 or E2F3 in Her2$^+$ cells decreased centrosome amplification without significantly affecting cell cycle progression, whereas the overexpression of E2F1, E2F2, or E2F3 increased centrosome amplification in MCF10A mammary epithelial cells. Our results revealed that E2Fs affect the expression of proteins, including Nek2 and Plk4, known to influence the cell/centrosome cycles and mitosis. Downregulation of E2F3 resulted in cell death and delays/blocks in cytokinesis, which was reversed by Nek2 overexpression. Nek2 overexpression enhanced centrosome amplification in Her2$^+$ breast cancer cells silenced for E2F3, revealing a role for the E2F activators in maintaining centrosome amplification in part through Nek2.

The E2F transcription factors regulate various biological functions, such as cell cycle progression, DNA repair, apoptosis, centrosome duplication, and differentiation (1–8). Eight E2F proteins have been identified and are categorized as activators E2F1 through E2F3a and repressors E2F3b through E2F8 (9, 10). Rb hyperphosphorylation by G_1/S-phase cyclin/Cdk complexes releases the E2F activators, which bind promoters through consensus (T/C)TT(C/G)(G/C)CG(C/G) or noncanonical binding sites (11, 12) to activate a plethora of genes that regulate the aforementioned cellular activities (4, 13, 14). The E2Fs are deregulated and altered in most human cancers through various molecular mechanisms, including overstimulation of the G_1/S-phase cyclin/Cdks that hyperphosphorylate and inactivate the Rb family (15). Another mode of deregulation is by overexpression, such as that of E2F1 in breast, lung, and prostate cancers (16–26) and E2F3 in various cancers, including breast cancers (18, 26–31).

Deregulated expression of the E2Fs in breast cancers influences outcome of survival, since patients overexpressing E2F1 and cyclin A displayed shorter disease-free survival (16). In addition, breast cancer cells with molecular alterations affecting the Rb pathway or E2F overexpression display altered chemotherapeutic responses (32–36), including resistance to the Cdk4/Cdk6 inhibitor PD-0332991 (37, 38). Mouse models demonstrated the requirement for E2Fs in mammary carcinogenesis, since ablation of E2F1 and E2F3 suppressed Her2/Neu and Myc-induced mammary tumorigenesis (26, 39, 40). Thus, studying E2F functions may provide clues not only to understanding how mammary tumors initiate and progress but also to how breast cancer cells fail to respond to common therapies.

The E2Fs may influence breast carcinogenesis by signaling various abnormal phenotypes, including centrosome amplification, defined as the acquisition of three or more centrosomes within a cell (6, 7). Centrosome amplification may initiate and sustain breast cancers by actively generating aneuploidy and chromosome instability (41), a hypothesis that remains to be tested. The centrosome must duplicate once in each cell cycle to maintain normal centrosome numbers, achieved by cell cycle and centrosome-specific regulators (42, 43). Faithful centrosome licensing (regulated in part by the phosphorylation of nucleophosmin [NPM] by Cdk2 and Cdk4), duplication (regulated by various kinases, including Plk4), and maturation and separation (regulated in part by Nek2) are essential to establish spindle bipolarity at mitosis and faithful segregation of chromosomes following cytokinesis (42–44). Deregulated centrosome duplication or cytokinesis defects are two major mechanisms leading to centrosome amplification, which results in aberrant pseudobipolar and multipolar mitotic spindles, chromosome losses/gains, and aneuploidy (7, 45–47).

Although various cancer types display elevated centrosome amplification (48, 49), the relationship between centrosome amplification and tumorigenesis is best understood in breast cancers, since a significant fraction of premalignant lesions and many breast tumors exhibit centrosome defects, including defects in numbers (centrosome amplification) or structure (size changes) (50–54). A major gap in knowledge is identifying pathways directly signaling centrosome amplification. Identifying the roles/functions and sources of centrosomal/mitotic kinases in signaling centrosome amplification is important to breast cancer control, since the overexpression of 16 centrosomal/mitotic kinases in breast cancer, including Nek2 and Plk4, represents a molecular signature that strongly associates with poorly prognostic breast cancers (55). In fact, Nek2 and Plk4 are overexpressed in low-

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TABLE 1 Primer sequences

Method and primer	Orientation[a]	Sequence (5′–3′)
Real-time PCR		
Cyclin D1	F	GGC TGG GTC TGT GCA TTT CT
	R	AAC ATG CCG GTT ACT TGT TGG T
Cyclin E1	F	TGGATCTCTGTGTCCTGGATGTT
	R	CAAGGCCGAAGCAGCAA
E2F1	F	GTT TGG GCC GGG TTT TG
	R	GCA TTT CCC CAG CAA CCT T
E2F2	F	AGG GTG TCC CTT TTC CAC AGT A
	R	CTT GAC CAC CTC CCT CTT CCT
E2F3	F	GCA TGA CAA CTC GTG TGT ATG AGA
	R	CAA TTG CCA CCC GAC TTA CTC
Nek2	F	CGT GAG AGA CTA GCA GAG GAC AAA
	R	TCC GTT CCT TTA GCA AGC TGT AG
Plk4	F	TGC ATA GTG CTG CTT CTC CAA
	R	GAC CAA GTC CTT CAT TTG TAA CCA
Actin	F	CGA GGC CCA GAG CAA GAG
	R	CGT CCC AGT TGG TAA CAA TGC
ChIP assay		
Nek2	F	TTG GCG ATC TCT ATC AGA GGG
	R	AAA GTG TCA CTA GGC AAC CGC
Plk4	F	AGT GTC CCG AGG CAC TGC GGC TT
	R	AGA TAA CCG CCA TCC CCT TGG A
siRNA analysis		
E2F1_2		AGCAAAUCAAAGUGCAGAUUGGAGGGU
E1F1_4		CUCUGGAAACCCUGGUCCCUCCAAGCC
E2F3a		UGAGGAUCUGGAUGUACGCUU
E2F3a_4		GUUCGUGGUGAGGAUCUGGAUGUACGC
Cyclin D1		CAAGAAUUACAUAGCCAAGAUGUGCAA
Site-directed mutagenesis		
Nek2 m1	F	CCT CTC TCC ATC CCT CCG TTT GGC TTA GC
	R	CGG AGG GAT GGA GAG AGG AAG CGG CAG
Plk4 m1	F	CAG CAA TCC ATC CCG AGC TAC CGC GTT AGA GC
	R	GGT AGC TCG GGA TGG ATT GCT GAA AGA ACG
Plk4 m2	F	GCT ACC GAT TTA GAG CAG GGC AGG GCA GG
	R	CTG CTC TAA ATC GGT AGC TCG GGC GGG
Plk4 m3	F	CGT TAG AAT AGG GCA GGG CTA CCT CC
	R	CCT GCC CTA TTC TAA CGC GGT AGC TCG G
Plk4 m4	F	GGG CAG GAT TAC CTC CCA CTT CTC CAA GG
	R	GGG AGG TAA TCC TGC CCT GCT CTA ACG C

[a] F, forward; R, reverse.

prognosis breast cancer molecular subtypes, individually associating with accelerated time-to-metastasis and time-to-relapse of breast cancer patients (56). Major unanswered questions regarding the role of the E2Fs in centrosome amplification are addressed in the present study, and we provide direct evidence that the E2F activators induce and maintain centrosome amplification in breast cancer cells and that Nek2 drives centrosome amplification downstream of the E2F3 activator.

MATERIALS AND METHODS

Cell culture. All cell lines were obtained from the ATCC (Manassas, VA) or from collaborators. The culture conditions for MCF10A, HCC1954, SKBR3, and JIMT1 cells have been described (57, 58). For serum starvation, cells were grown in media containing 0.2% fetal bovine serum (FBS) for 72 h. To develop stably silenced E2F cell populations, 2 μg of puromycin/ml was added to the media and 50 μg of hygromycin/ml was added in the media to develop MCF10A cells overexpressing E2Fs. Both puromycin and hygromycin were added to develop HCC1954 and JIMT1 cells stably knocked down for E2F3 (shE2F3) and overexpressing green fluorescent protein (GFP)-tagged Nek2 (shE2F3; GFP-Nek2).

Real-time PCR analysis. Total RNA was isolated using TRIzol according to the manufacturer's protocol (Invitrogen, Grand Island, NY), and 2 μg of RNA was used to synthesize cDNA according to the manufacturer's protocol (Promega, Madison, WI). Then, 2 μl of 1:10-diluted cDNA was used for real-time PCR with iQ SYBR green Supermix (170-8880; Bio-Rad, Hercules, CA). Actin was used as an internal control, and the primer sequences are presented in Table 1.

Transfection of siRNAs and BrdU incorporation assay. Lipofectamine 2000 (11668-019; Invitrogen), along with 200 pmol of each E2F or cyclin D1 small interfering RNA (siRNA) constructs (Integrated DNA Technologies, Coralville, IA) or 5 μl of silencer negative-control siRNA 1 (50 μM, AM4611; Ambion, Grand Island, NY), was used. The primer sequences used for these experiments are presented in Table 1. Bromodeoxyuridine (BrdU) incorporation assay was performed according to our published protocols (59). The percentages of BrdU-positive cells in a population of at least 500 cells were calculated.

Generation of shE2F and shE2F3; GFP-Nek2 cell clones. To generate stably silenced E2F cells, we used short hairpin RNA (shRNA)-mediated knockdown based on the lentiviral vector pLKO.1-puro (Addgene, Cambridge, MA). At 24 h after the second infection, cells were subjected to selection with 2 μg of puromycin/ml, and cell populations were obtained. Nek2 was subcloned into the pMONO-hygro-GFP plasmid (Invivogen, San Diego, CA) by the Emory DNA Custom Cloning Core Facility. pMONO-hygro-GFP-Nek2 was transfected using Lipofectamine 2000 into HCC1954 and JIMT1 cells stably downregulated for E2Fs.

Cell cycle analysis. To analyze the cell cycle, we used fluorescein isothiocyanate BrdU/7-AAD flow cytometry kits (catalog no. 57891; BD Pharmingen, San Jose, CA). Briefly, 2×10^6 to 3×10^6 cells were plated on a 100-mm culture dish and cultured in serum starvation media (0.2% serum) for 72 h, released to 10% FBS-containing media, and harvested at 0, 12, 18, and 24 h. Before harvesting, the cells were pulse-labeled with 10 μM BrdU for 30 min at 37°C. The cells were processed and immunostained according to the manufacturer's protocol, acquired in a BD LSRII apparatus using flow cytometry, and analyzed with FlowJo software (Tree Star, Ashland, OR).

Immunostaining. Centrosome amplification assays were done by plating cells on a four-well chamber slide and fixation in 4% paraformaldehyde for 10 min. Cells were permeabilized in 0.1% NP-40 for 10 min after being washed three times with phosphate-buffered saline. Cells were blocked in 10% normal goat serum (Invitrogen) for 1 h, following overnight primary antibody incubation against pericentrin (ab4448; Abcam, Cambridge, MA). Two hundred cells were counted, and cells with ≥3 pericentrin-positive cells are presented as percentages. For binucleation assays, the cytoskeleton was localized with α-tubulin antibody (sc-32293; Santa Cruz Biotechnology). Alexa Fluor-conjugated antibodies (catalog nos. A11008, A11001, or A21069; Invitrogen) were used as secondary antibodies. For counterstaining, DAPI (4′,6′-diamidino-2-phenylindole) at 1 mg/ml was applied. Two hundred cells were counted, or images were obtained at ×40 magnification using a Zeiss Axioplan-2 fluorescence microscope.

Chromatin immunoprecipitation (ChIP) assay. Cells were plated on a 150-mm culture dish, and when they were 80 to 90% confluent the cells were cross-linked with 1% formaldehyde for 10 min on a shaker and quenched by adding 0.156 M glycine. After two washing steps, the cells were scraped off the plate for harvesting, and the rest of steps were followed as described previously (60). The following antibodies were used: E2F1 (3742; Cell Signaling, Danvers, MA), E2F2 (sc-633; Santa Cruz Biotechnology), and E2F3 (sc-878; Santa Cruz Biotechnology). Normal rabbit IgG antibody (2729; Cell Signaling) was used as a negative control. The sequences used in this assay are shown in Table 1.

Luciferase assay. Approximately 1 and 1.2 kb of human Nek2 and Plk4 proximal-promoter regions were cloned into pGL3-Basic plasmid (E1751; Promega) and sequenced. E2F binding site mutants on Nek2 and Plk4 promoter regions were generated by site-directed mutagenesis using Phusion DNA polymerase (M0530; New England Biolabs) with the mutant primers listed in Table 1. Her2$^+$ cell lines were cotransfected with either pGL3-Nek2, pGL3-Plk4 or mutant constructs along with pRL-CMV (E2261; Promega) as an internal control using TransIT-2020 transfection reagent (MIR5400; Mirus, Madison, WI) for 48 h, and cells were assayed for promoter activity by using a dual luciferase kit (E1910; Promega).

Western blotting. Western blotting was performed according to our published protocols (59, 61, 62). The following primary antibodies were used in this experiment: E2F1 (3742; Cell Signaling), E2F2 (sc-633; Santa Cruz Biotechnology), E2F3 (sc-878; Santa Cruz Biotechnology), cyclin D1 (2922; Cell Signaling), cyclin E (sc-481; Santa Cruz Biotechnology), Nek2 (610593; BD Biosciences, San Jose, CA), phospho-NPM^{T199} (3541; Cell Signaling), and Plk4 (ab56752; Abcam). β-Actin antibody (4970; Cell Signaling) was used as a loading control. For secondary antibodies, either goat anti-rabbit antibody (sc-2004) or goat anti-mouse antibody (sc-2005) conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was used. Signals were detected by using a Lumigen TMA-6 reagent (Lumigen, Inc., Southfield, MI). ImageJ software (National Institutes of Health, Bethesda, MD) was used to quantify protein levels.

Live-cell image analysis. HCC1954 cells transduced with pLKO.1, shE2F3, or shE2F3; GFP-Nek2 (1×10^4 to 2×10^4 cells/well) were plated on an eight-chambered #1.5 German coverglass system (155409; Thermo Scientific). Cells were placed in Perkin-Elmer Ultra-View microscope (Perkin-Elmer, Waltham, MA) set at 37°C and 5% CO_2, with a differential interference contrast filter, and live-cell images were captured every 5 min for 45 to 48 h under a 10× objective lens and compiled into movies for analysis. All image capture and analysis was done using Volocity 3D image analysis software (Perkin-Elmer).

Bioinformatic analysis. The Cancer Genome Atlas (TCGA) RNASeq data from 922 breast cancer adenocarcinoma (BRCA) patients (Illumina HiSeq RNASeqv2 level 3 RSEM normalized level 3 gene data) were downloaded from the Broad Institute Firehose Standard Data set portal (63). Clinical metadata on the same data set were also obtained from the same source. Clinical subtypes were taken from previously published analyses of TCGA BRCA samples (64). Pearson correlation coefficients and associated *P* values were computed for E2F1, E2F2, and E2F3 relative to NEK2 and PLK4 for the entire data set and for clinically relevant subtypes. Scatterplots were generated by two-gene RNAseq analysis of the provisional TCGA breast data set on the cBio Cancer Genomics Portal website (65).

Statistical analysis. The Student *t* test was applied to compare significance between groups, and *P* value of <0.05 are indicated by an asterisk. For promoter analysis, we applied the Mann-Whitney U test (nonparametric test). For live cell imaging analysis, either the chi-square test or the Fisher exact test was applied to compare the proportion of each type between each pair of cell lines, and the SAS statistical package (v9.3; SAS Institute, Inc., Cary, NC) was used for analyses with a significance level of 0.05.

RESULTS

The E2F activators and proteins regulating the cell and centrosome cycles are deregulated in Her2$^+$ breast cancer cells harboring centrosome amplification. We selected Her2$^+$ breast cancer cells that display centrosome amplification to establish whether E2F downregulation diminishes this abnormal phenotype. Using data presented in Fig. 1a and in two previous publications (56, 62), we screened cell lines of different molecular subtypes, including Her2$^+$, triple-negative (ER$^-$ PR$^-$ Her2$^-$) and luminal (ER$^+$ Her2$^+$ or Her2$^-$) subtypes, and found centrosome amplification in roughly 50% of Her2$^+$ breast cancer cells. Although there is significant elevation of centrosome amplification in Her2$^+$ cells (SKBR3, HCC1954, and JIMT-1), other Her2$^+$ cells (BT4T4, HCC1419, and HCC1569), and luminal ER$^+$ PR$^-$ Her2$^+$ (MDA-MB-361), luminal ER$^+$ PR$^+$ Her2$^-$ (MCF7 and T47D), and triple-negative (MDA-MB-231 and MDA-MB-468) cell lines do not display centrosome amplification (56, 62). MCF10A cells are used as a control, since they are nontransformed, immortalized human mammary epithelial cells that lack the p16^{INK4A} and p14^{INK4B} tumor suppressors and display normal p53 activity (66, 67). The genetic characteristics and origins of these cell lines have been described (58, 66). To investigate whether the E2F activators

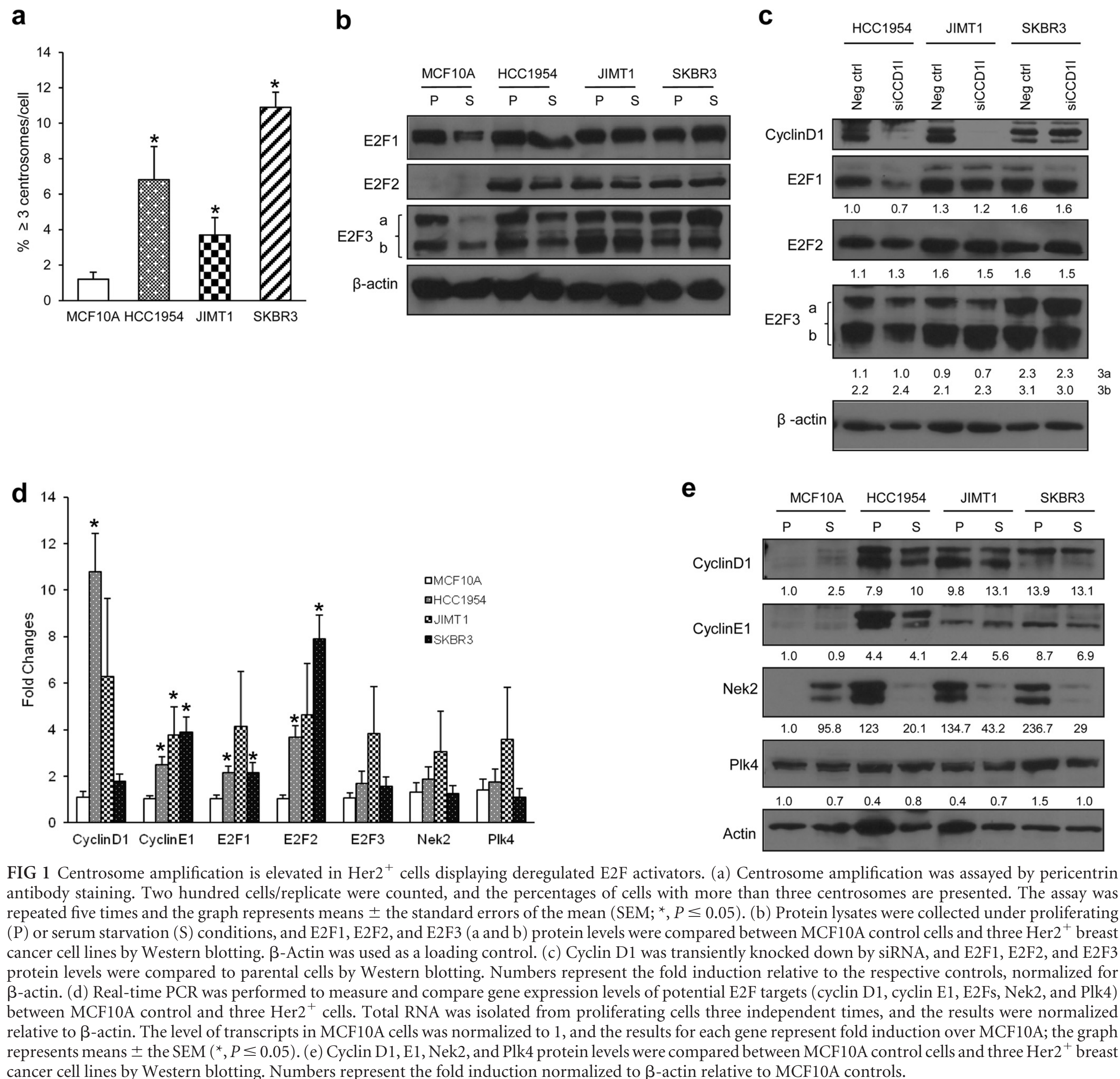


FIG 1 Centrosome amplification is elevated in Her2$^+$ cells displaying deregulated E2F activators. (a) Centrosome amplification was assayed by pericentrin antibody staining. Two hundred cells/replicate were counted, and the percentages of cells with more than three centrosomes are presented. The assay was repeated five times and the graph represents means ± the standard errors of the mean (SEM; *, $P \leq 0.05$). (b) Protein lysates were collected under proliferating (P) or serum starvation (S) conditions, and E2F1, E2F2, and E2F3 (a and b) protein levels were compared between MCF10A control cells and three Her2$^+$ breast cancer cell lines by Western blotting. β-Actin was used as a loading control. (c) Cyclin D1 was transiently knocked down by siRNA, and E2F1, E2F2, and E2F3 protein levels were compared to parental cells by Western blotting. Numbers represent the fold induction relative to the respective controls, normalized for β-actin. (d) Real-time PCR was performed to measure and compare gene expression levels of potential E2F targets (cyclin D1, cyclin E1, E2Fs, Nek2, and Plk4) between MCF10A control and three Her2$^+$ cells. Total RNA was isolated from proliferating cells three independent times, and the results were normalized relative to β-actin. The level of transcripts in MCF10A cells was normalized to 1, and the results for each gene represent fold induction over MCF10A; the graph represents means ± the SEM (*, $P \leq 0.05$). (e) Cyclin D1, E1, Nek2, and Plk4 protein levels were compared between MCF10A control cells and three Her2$^+$ breast cancer cell lines by Western blotting. Numbers represent the fold induction normalized to β-actin relative to MCF10A controls.

(E2F1, E2F2, and E2F3a) are deregulated in Her2$^+$ breast cancer cells and drive centrosome amplification, their protein levels were analyzed in three ER$^-$ PR$^-$ Her2$^+$ breast cancer cell lines (henceforth referred to as Her2$^+$ cells): HCC1954, JIMT1, and SKBR3 (Fig. 1b). In proliferating MCF10A cells, E2F1 and E2F3 were robustly expressed. Unlike the decreases in E2F1 and E2F3a expression achieved under serum starvation of MCF10A cells, the E2F protein levels in Her2$^+$ cells were unaffected by serum starvation, showing deregulation of E2Fs. Since E2Fs are under the control of cyclin D1, the upregulation of which has been reported in many types of breast cancer cells (68), we investigated whether the deregulation of E2F proteins seen in Her2$^+$ cells is caused by deregulated cyclin D1. Thus, we transiently knocked down cyclin D1 by siRNA and measured the E2F levels. Cyclin D1 knockdown was efficient in HCC1954 and JIMT1 cells (Fig. 1c). The levels of E2F1, E2F2, or E2F3 were not significantly changed in cyclin D1 knockdown cells except for slight decreases of E2F1 in HCC1954 cells and of E2F3a in HCC1954 and JIMT1 cells. Nevertheless, the protein levels of E2Fs remained robust in the absence of cyclin D1. Her2$^+$ cell lines displaying deregulated E2F activators also displayed centrosome amplification (Fig. 1a). We chose various canonical or potential E2F targets known to play roles in regulating the cell and centrosome duplication cycles based on our previously published reports in which we screened premalignant mouse mammary epithelial lesions expressing K-Ras^{G12D} or K-Ras^{G12D} and c-Myc for the expression of most known molecules

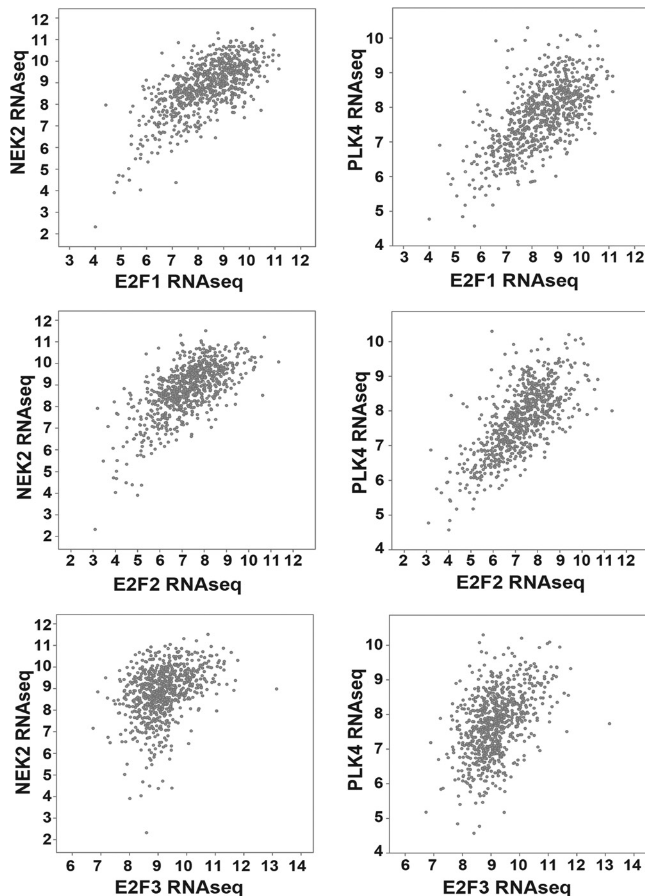


FIG 2 Scatterplots of RNASeq data for NEK2 and PLK4 relative to E2F factors. Scatterplots were generated using the cBioPortal Cancer Genomics website (www.cbioportal.org) analyzing the provisional complete set of TCGA BRCA samples (last accessed 14 January 14). Each axis shows the $\log_2$ value of the RNASeq-V2-RSEM mRNA data from TCGA.

regulating the cell and centrosome cycles (69). Those studies showed conservation in signaling between premalignant lesions expressing such oncogenes and Her2$^+$ breast cancer cells (62). We found significantly elevated mRNA levels of cyclin D1, cyclin E1, E2F1, and E2F2 in Her2$^+$ cells compared to MCF10A cells, whereas the levels of E2F3, Nek2, and Plk4 were slightly, but not significantly, increased in HCC1954 and JIMT1 cells (Fig. 1d). Western blots confirmed real-time PCR data showing high levels of cyclin D1 and cyclin E1, and there were no major changes in Plk4 with the exception of its overexpression in SKBR3 cells (Fig. 1e). In contrast to the real-time PCR data, Nek2 protein was consistently overexpressed in all three Her2$^+$ breast cancer cell lines relative to MCF10A. Our experiments show that the specific breast cancer cell lines used in the present study display centrosome amplification, deregulated E2F activators, and canonical and potential E2F transcriptional targets.

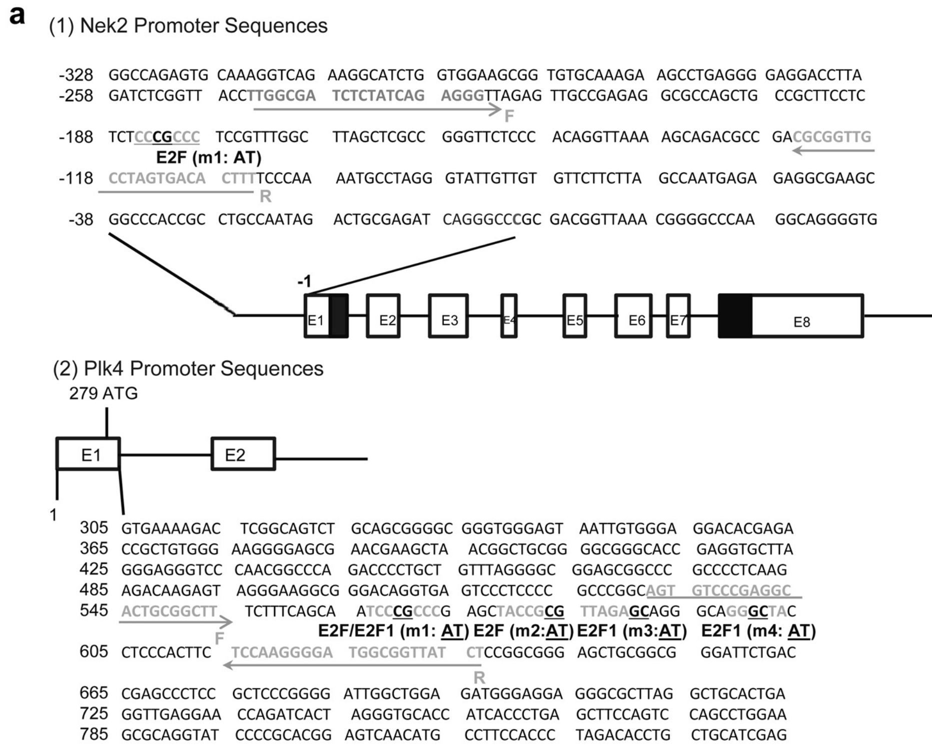
Nek2 and Plk4 are strongly correlated with E2F factors in breast cancer patients. To determine whether the relationship between E2F family transcription factors and Nek2/Plk4 is observed in breast cancer patients, we mined publicly available RNAseq data from the TCGA project in BRCA data sets. We performed scatterplot analysis of Nek2 and Plk4 against E2F1, E2F2, and E2F3 using the cBio Cancer Genomics Portal (www.cbioportal.org) and observed a strong correlation of each E2F factor with their target genes (Fig. 2). To quantify the correlation, we downloaded normalized gene RNASeq data from the TCGA Data Coordination Center (63) and computed the Pearson correlation coefficients and corresponding *P* values (Table 2) for all 922 TCGA patients, as well as for basal, Her2$^+$, luminal A, and luminal B subsets based on published classifications (64). Correlations were highly statistically significant, except for the Her2$^+$ subtype, suggesting that E2F family transcription factors regulate Nek2 and Plk4 in breast cancer patients.

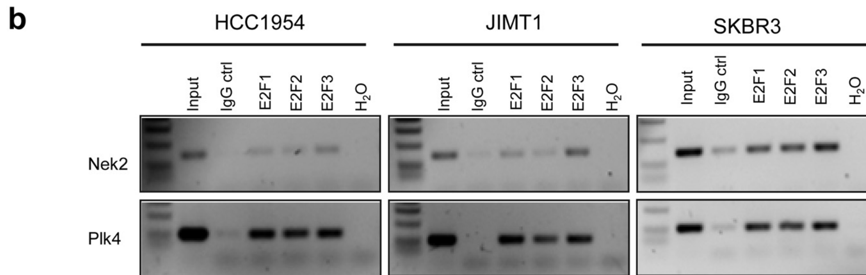
Plk4 is a direct transcriptional target of the E2F activators. Cyclin D1, cyclin E1, and the E2F activators are known targets of the E2Fs (4, 13, 70). Although the data presented in Table 2 and Fig. 2 show strong correlations between overexpressed E2Fs and elevated Nek2/Plk4 transcripts in breast cancer, it is unknown whether the E2Fs are direct regulators of Nek2 and Plk4. Conserved transcription factor binding finder (CONFAC) analysis (71, 72) predicted one putative E2F site on the Nek2 promoter (70) and four putative E2F binding sites between exons 1 and 2 of Plk4 (Fig. 3a). To elucidate whether E2F1, E2F2, and E2F3 associate with regions on the Nek2 and Plk4 promoters, we used a ChIP assay. The data showed that all three E2F activators bound the predicted E2F binding sites in the Nek2 and Plk4 promoters (Fig. 3b). ChIP analysis in shE2F1 or shE2F3 cells showed no decreases in the occupancy of the E2F site by E2F1, E2F2, and E2F3 on the Nek2 promoter (Fig. 3c). On the other hand, downregulation of E2F1 significantly decreased binding of E2F1, E2F2, and E2F3 to the Plk4 promoter regions. E2F3 downregulation led to slight but not significant decreases in E2F occupancy in HCC1954 cells and significant decreases in SKBR3 cells. In addition, a luciferase assay

TABLE 2 Correlation of NEK2 and PLK4 with E2F factors in TCGA BRCA samples[a]

TCGA sample group (*n*) and E2F factor	NEK2		PLK4	
	Correlation	*P*	Correlation	*P*
All BRCA (922)				PLK4
E2F1	**0.60**	**3.22E–92**	**0.54**	**6.13E–71**
E2F2	**0.51**	**1.45E–62**	**0.50**	**2.21E–58**
E2F3	**0.37**	**1.65E–31**	**0.43**	**8.38E–44**
Basal (96)				
E2F1	**0.43**	**1.04E–05**	**0.19**	**6.28E–02**
E2F2	**0.35**	**5.27E–04**	**0.19**	**5.82E–02**
E2F3	0.17	9.97E–02	**0.30**	**3.24E–03**
Her2 (55)				
E2F1	0.07	6.07E–01	0.00	9.94E–01
E2F2	–0.08	5.45E–01	–0.05	7.11E–01
E2F3	–0.03	8.02E–01	–0.23	9.80E–02
Luminal A (230)				
E2F1	**0.45**	**8.18E–13**	**0.57**	**4.02E–21**
E2F2	**0.46**	**1.86E–13**	**0.51**	**1.28E–16**
E2F3	**0.21**	**1.23E–03**	**0.32**	**1.04E–06**
Luminal B (126)				
E2F1	**0.31**	**4.97E–04**	**0.48**	**1.24E–08**
E2F2	**0.27**	**2.69E–03**	**0.47**	**3.42E–08**
E2F3	0.13	1.46E–01	**0.20**	**2.38E–02**

[a] Values indicated in boldface are significantly different.





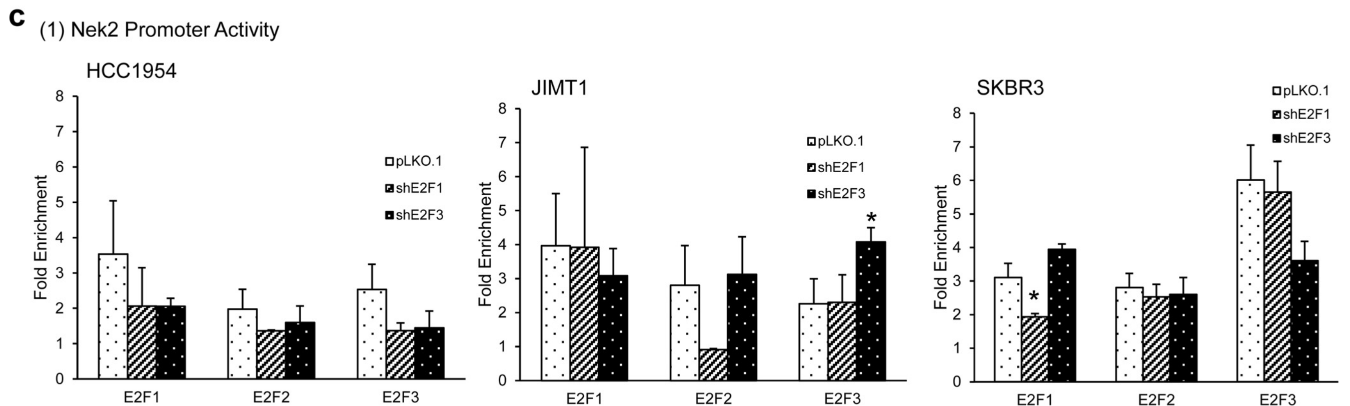
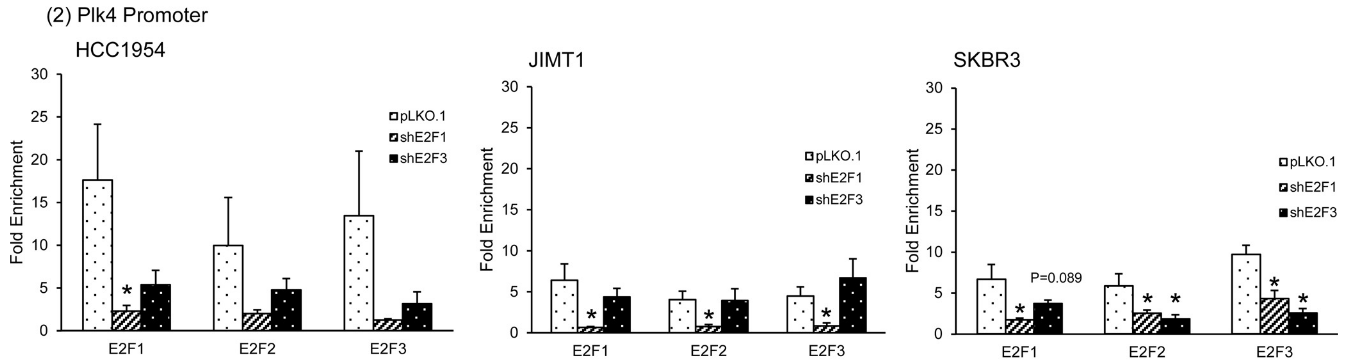


FIG 3 The E2F activators bind the Nek2 and Plk4 promoter regions and increase their promoter activities. (a) Location of canonical E2F binding sites adjacent to the Nek2 and Plk4 promoters, as predicted by the CONFAC program. The primers used for amplification and mutant E2F promoter sequences are also indicated. (b) E2F1, E2F2, or E2F3 antibodies were used to immunoprecipitate chromatin displaying potential E2F binding sites located in proximity to the Nek2 and Plk4 promoters in HCC1954, JIMT1, and SKBR3 cells. A fraction of chromatin was used as a nonimmunoprecipitated input control. Normal rabbit IgG was used as a negative control for the ChIP assay, and H_2O was used as control for PCR. (c) ChIP assay on shE2F1 and shE2F3 cells. The *x* axis indicates the antibody used for immunoprecipitation, while the *y* axis indicates the fold enrichment relative to IgG controls. (d) Nek2 and Plk4 promoter activities were measured using dual-luciferase assay in three $Her2^+$ cell lines, as well as MCF10A cells. The graph represents means ± the SEM (*, $P \leq 0.05$).



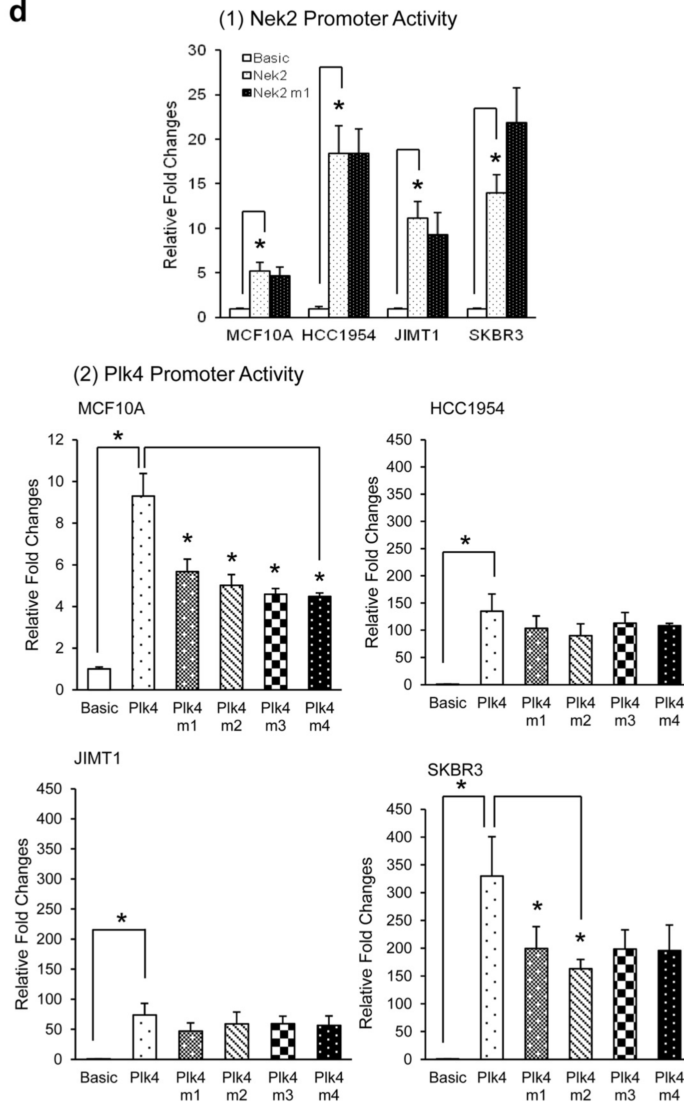


FIG 3 continued

showed higher Nek2 promoter activity in Her2$^+$ cells (2- to 4-fold) compared to that of MCF10A control (Fig. 3d1). In regard to the Plk4 promoter, the activity was 15- to 30-fold higher in Her2$^+$ cells relative to MCF10A cells (Fig. 3d2). To analyze this further, E2F binding site mutants of the Nek2 (Nek2 m1) and Plk4 promoter (Plk4 m1, m2, m3, and m4) were created by site-directed mutagenesis, by changing the core bases CG to AT. We did not detect decreased luciferase activity of the E2F binding site mutant on the Nek2 promoter (Fig. 3d1). We cloned more than 3 kb of the promoter region into the luciferase construct to detect

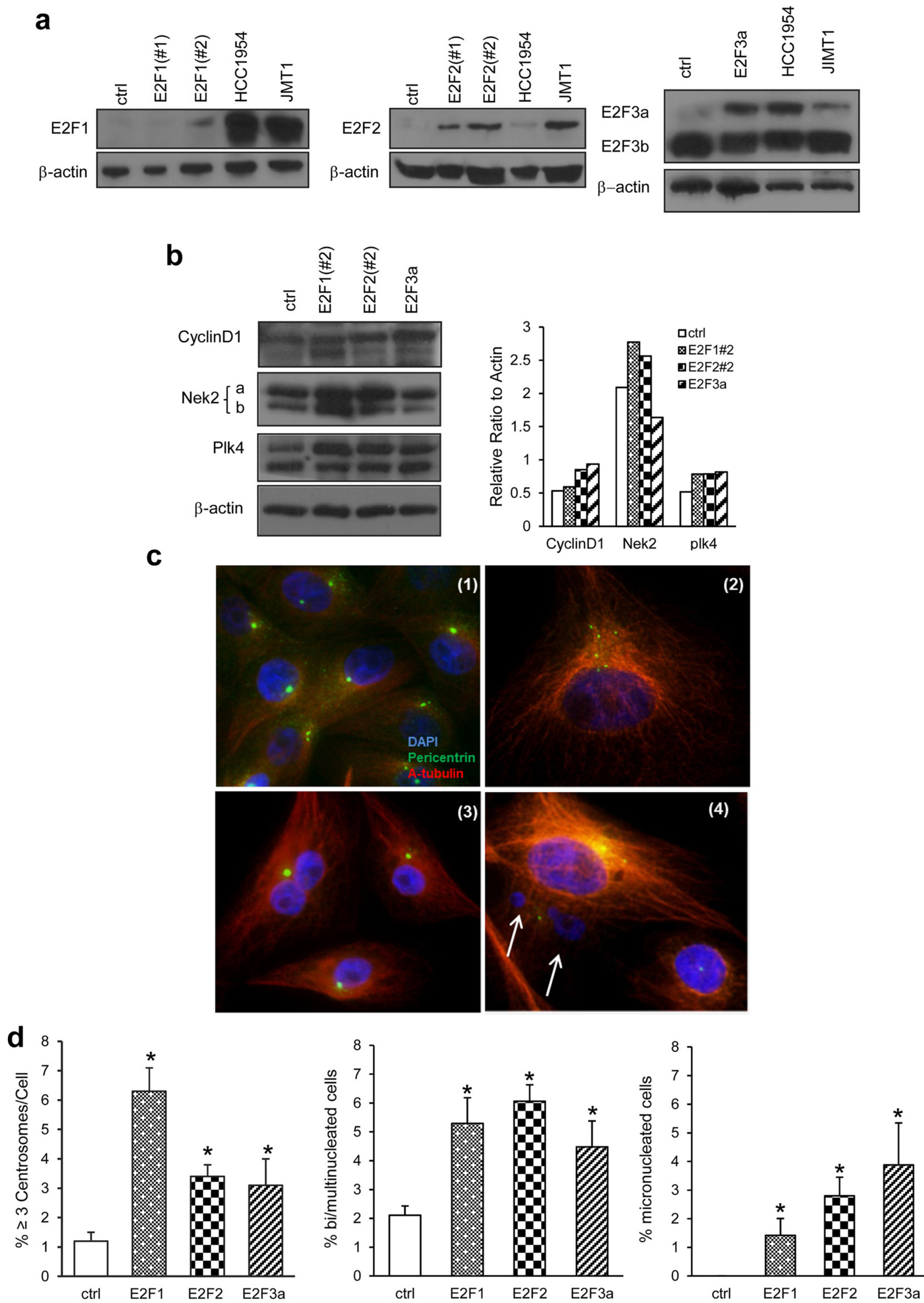


FIG 4 Ectopic expression of E2F1, E2F2, or E2F3a results in overexpression of centrosome regulators, centrosome amplification and chromosome instability. (a) Cells overexpressing human E2F1, E2F2, or E2F3a (pBH-*E2F1*, pBH-*E2F2*, or pBH-*E2F3a*, respectively) were generated in MCF10A cells, and the E2F levels were measured by Western blotting. (b) Canonical and potential targets of E2Fs—cyclin D1, Nek2, and Plk4—were analyzed by Western blotting, and their levels were quantified. Nek2a and Nek2b were quantified in the same graph. (c) Microscope images of pericentrin/α-tubulin/DAPI staining (1, normal number of centrosomes in MCF10A cells; 2, centrosome amplification; 3, binucleation; 4, micronucleation) in E2F overexpressing MCF10A cells. Pictures were taken with a Zeiss Axioplan-2 microscope under ×40 magnification. (d) Centrosome amplification was assayed by pericentrin staining, binucleation by α-tubulin, and micronucleation by DAPI. The percentage of cells with three or more centrosomes was calculated in 200 cells per replicate per group. The assay was repeated three times and the graph was represented by mean percentages ± the SEM (*, $P \leq 0.05$).

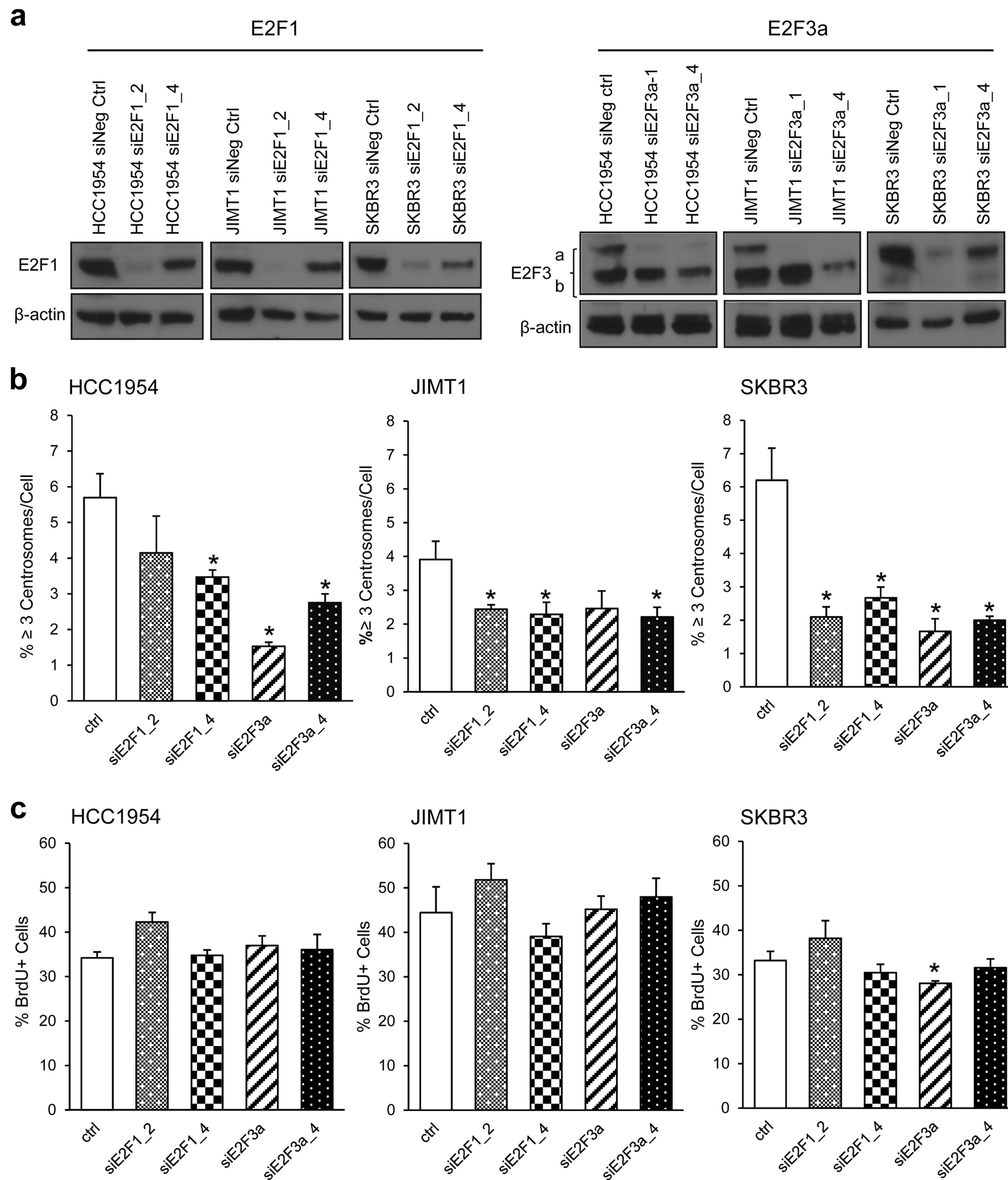
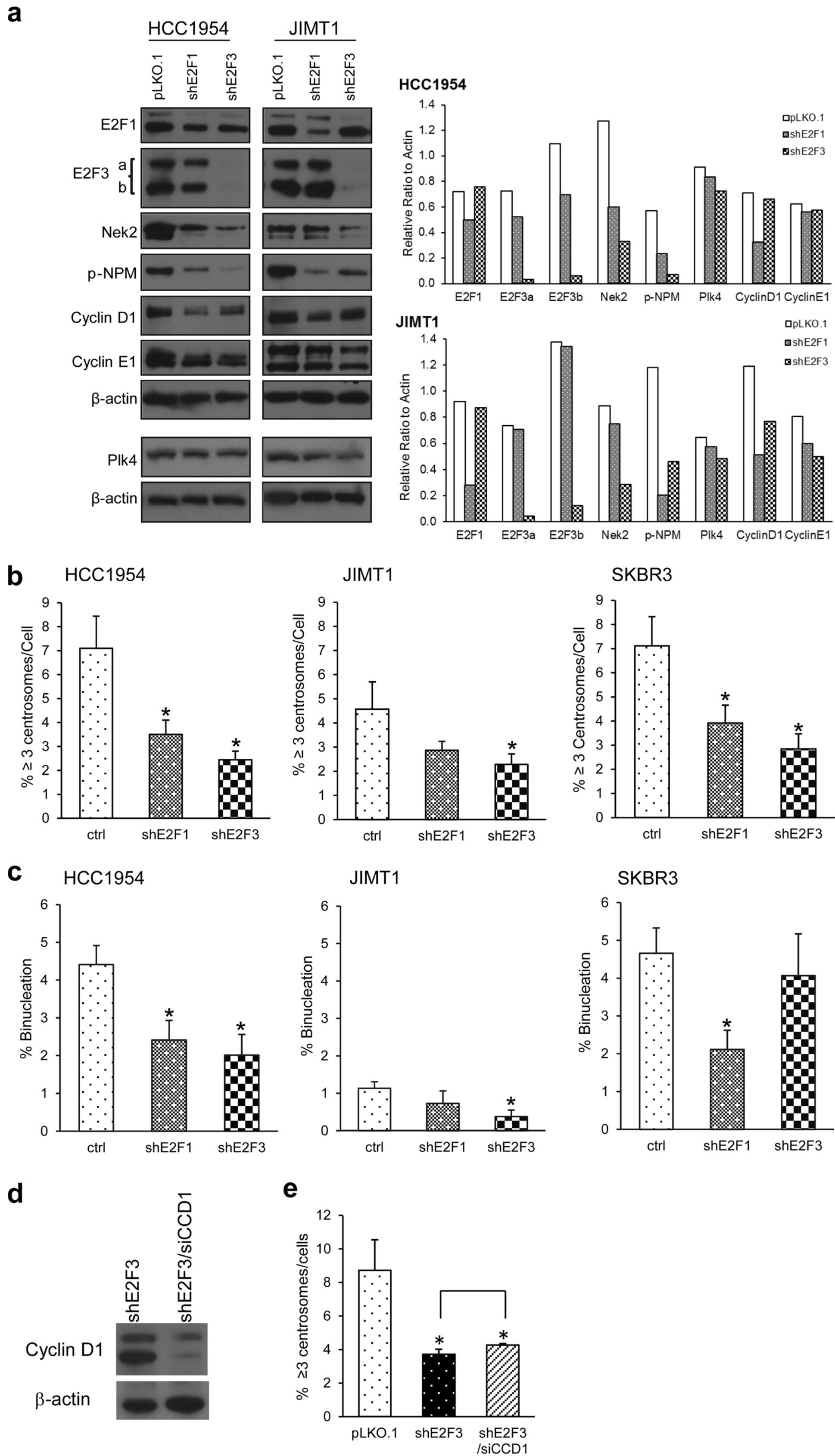


FIG 5 Transient knockdown of E2F1 or E2F3a suppresses centrosome amplification without greatly affecting DNA replication. (a) siRNA-mediated gene silencing was used to transiently knockdown E2F1 and E2F3a in three Her2^{+} cells. Two independent siRNAs sequences against E2F1 (siRNAE2F1_2 and siRNAE2F1_4) or E2F3a (siRNAE2F3a and siRNAE2F3a_4) were used to transfect target cells. Western blotting was performed to detect protein levels; β-actin served as a loading control. (b) Cells were transfected with the indicated control or siRNA against E2F1 or E2F3a. Graphs represent the percentages of cells with more than three centrosomes (localized by pericentrin antibody staining). Each replicate was done in a population of at least 200 cells. The assay was repeated three times, and the graph shows the means ± the SEM (*, $P \leq 0.05$). (c) A BrdU incorporation assay was performed to measure DNA synthesis in cells silenced for E2F1 or E2F3a. Cells were pulsed with BrdU and processed for BrdU staining. Graphs represent BrdU^{+} cells in a population of cells. A population of 500 cells was counted per group per replicate. Three independent experiments were performed, and the graph shows the means ± the SEM (*, $P \leq 0.05$).

nonconsensus E2F sites but could not detect additional sites or the high luciferase expression detected with the minimal promoter (data not shown). In contrast, all four mutations of E2F binding sites on the Plk4 promoter significantly decreased its promoter activities (ca. 40 to 50% that of parental construct) in MCF10A cells. Overall, Plk4 promoter activities were decreased ca. 15 to 40% in mutant constructs compared to the wild type in three Her2^{+} cells, but most of them were not statistically significant except Plk4 mutant 1 and 2 constructs in SKBR3 (Fig. 3d2). These data imply that mutation of single E2F binding sites on the Plk4 promoter was not enough to decrease E2F-dependent regulation of Plk4 transcription in all breast cancer cells. Together, these data


a
HCC1954
JIMT1
pLKO.1
shE2F1
shE2F3
E2F1
E2F3
a
b
Nek2
p-NPM
Cyclin D1
Cyclin E1
β-actin
Plk4
β-actin
HCC1954
JIMT1
Relative Ratio to Actin
E2F1
E2F3a
E2F3b
Nek2
p-NPM
Plk4
CyclinD1
CyclinE1
b
HCC1954
JIMT1
SKBR3
% ≥ 3 centrosomes/Cell
% ≥ 3 Centrosomes/Cell
ctrl
shE2F1
shE2F3
c
HCC1954
JIMT1
SKBR3
% Binucleation
ctrl
shE2F1
shE2F3
d
shE2F3
shE2F3/siCCD1
Cyclin D1
β-actin
e
% ≥3 centrosomes/cells
pLKO.1
shE2F3
shE2F3
/siCCD1

show that Plk4 is under direct E2F transcriptional regulation, whereas Nek2 is not.

E2F overexpression induces centrosome amplification and chromosome instability in immortalized mammary epithelial cells. We have reported that MCF10A cells have a low percentage of centrosome amplification (56, 62, 69). To address whether the overexpression of E2F1, E2F2, or E2F3 is sufficient to induce centrosome amplification in MCF10A cells, we developed stable populations of MCF10A cells overexpressing E2F1, E2F2, or E2F3a. Overexpression of the E2Fs was confirmed by Western blotting (Fig. 4a). This analysis revealed that E2F1, E2F2, and E2F3a are overexpressed relative to MCF10A parental controls at similar (E2F2 and E2F3a) or lower (E2F1) levels relative to Her2$^+$ cells. The data presented in Fig. 1d and e revealed the overexpression of various cell and centrosome regulatory proteins, including Nek2 and cyclin D1 in all Her2$^+$ cells, and significant overexpression of Plk4 in SKBR3 cells. Thus, we addressed whether single overexpression of E2Fs in MCF10A cells increased protein level of above targets. Populations expressing the highest E2F levels were chosen for this analyses and showed that MCF10A cells expressing single E2F1, E2F2, or E2F3a displayed upregulation of cyclin D1, Nek2, and Plk4 (Fig. 4b). Based on these observations, we further analyzed E2F-overexpressing cells to measure centrosome amplification by pericentrin (Fig. 4c2) and to quantify binucleated (Fig. 4c3) and micronucleated cells (Fig. 4c4) by α-tubulin/DAPI. Binucleation, an intermediate to tetraploidy (46) (Fig. 4c3), and micronucleation, a measure of whole chromosome or chromosome fragment losses (73) (Fig. 4c4), are measures of chromosome instability (73–75). Our results demonstrate that ectopic E2F expression in MCF10A cells significantly elevated frequencies of centrosome amplification, binucleation, and micronucleation relative to control cells (Fig. 4d). The results confirmed that Nek2 and Plk4 levels are under direct or indirect E2F activator control and demonstrate that single E2F activators are sufficient to trigger generators of aneuploidy and chromosome instability.

Transient silencing of the E2F activators in Her2$^+$ breast cancer cells decreases centrosome amplification without significantly affecting DNA replication. Having demonstrated that E2F overexpression triggers centrosome amplification in nontransformed mammary epithelial cells, we addressed whether the E2F activators maintain centrosome amplification in Her2$^+$ breast cancer cells by transiently transfecting siRNAs to silence E2F1, E2F2, and E2F3a. We screened several siRNA constructs against E2F2 and did not achieve downregulation of E2F2 protein (data not shown). Two E2F1 siRNA constructs, siE2F1_2 and siE2F1_4, knocked down E2F1 efficiently in all three Her2$^+$ cell lines (Fig. 5a, left panel). For E2F3, we designed siRNA constructs specifically targeting the E2F3a activator, and both constructs efficiently knocked down E2F3a in all three Her2$^+$ cell lines (Fig. 5a, right panel). Transient downregulation of E2F1 and E2F3a was accompanied by decreased centrosome amplification in the three Her2$^+$ cell lines (Fig. 5b). Since E2Fs play a significant role in controlling DNA replication and to establish whether the cause of the decrease in centrosome amplification was a result of a general block in DNA replication, we measured the percentage of cells in S phase with a BrdU incorporation assay. Neither knockdown of E2F1 nor knockdown of E2F3a affected DNA replication in Her2$^+$ cells relative to cells transfected with control siRNA (Fig. 5c). The data indicate that transient knockdown of E2F1 and E2F3a diminishes centrosome amplification and that a block in S-phase progression is not a likely mechanism leading to reduced centrosome amplification. These data demonstrate a role for E2F1 and E2F3a in maintaining centrosome amplification in Her2$^+$ breast cancer cells.

Stable E2F knockdowns in Her2$^+$ breast cancer cells decrease centrosome amplification and binucleation but do not significantly affect cell cycle progression. To explore the roles of E2Fs in centrosome amplification more closely, stable E2F1 and E2F3 knockdowns were generated in JIMT1, SKBR3, and HCC1954 cells using shRNA-mediated silencing techniques. E2F1 knockdown was partial, whereas E2F3 knockdown was almost complete (Fig. 6a). Next, we investigated whether knockdown of E2F1 and E2F3 in HCC1954 and JIMT1 cell lines impacts proteins involved in the centrosome duplication cycle. Silencing E2F1 or E2F3 led to decreased cyclin D1 levels in both cell lines (Fig. 6a), whereas changes in cyclin E1 or Plk4 were small. Silencing E2F1 or E2F3 also led to lower levels of Nek2 and phospho-NPM^{T199}, a known phosphorylation target of Cdk2 and Cdk4 (59, 76, 77). Overall, these results confirm that the E2F activators modulate expression of central regulators of the cell and centrosome cycles, which may signal centrosome amplification in Her2$^+$ breast cancer cells.

Mirroring transient knockdowns, stable knockdown of E2F1 or E2F3 suppressed centrosome amplification in all cell lines (Fig. 6b). To establish whether silencing of E2Fs diminished binucleation, we calculated its percentages in cells silenced for E2F1 and E2F3 (Fig. 6c). Noticeably, JIMT1 cells had a lower extent of binucleation. Silencing E2F1 significantly decreased the percentage of binucleated cells in HCC1954 and SKBR3 cells. On the other hand, silencing E2F3 significantly decreased the percentages of binucleated cells in HCC1954 and JIMT1 cells. Consistent with Fig. 1c, where transient knockdown of cyclin D1 did not affect the protein expression levels of E2F3, transient knockdown of cyclin D1 in shE2F3 cells did not decrease CA further compared to that of shE2F3 alone (Fig. 6d and e). These data imply that E2Fs and cyclin D1 do not cooperate in maintaining CA in Her2$^+$ cells. To establish whether the decreases in binucleation and centrosome amplification were due to blocks in the cell cycle, we performed a flow cytometry-based DNA replication assay that measures BrdU incorporation and calculated the percentages of cells in various phases of the cell cycle (Table 3). Under proliferating conditions,

FIG 6 Her2$^+$ cells stably knocked down for E2F1 or E2F3 display lower levels of cyclin D1, Nek2, Plk4, and p-NPM^{199} and suppress centrosome amplification and binucleation. (a) E2F1 or E2F3 were stably downregulated using shRNA-mediated silencing technique in HCC1954 and JIMT1 cells. Potential E2F target protein levels (cyclin D1, Nek2, Plk4, and p-NPM^{199}) were compared in these cell lines by Western blotting and quantified (right panel). (b) Centrosome amplification was assayed by immunostaining centrosomes with pericentrin antibodies. Two hundred cells per group per replicate were counted. The assay was repeated five times, and the graph shows the mean percent cells with three or more centrosomes ± the SEM (*, $P \leq 0.05$). (c) A binucleation assay was performed by immunostaining the cytoskeleton with α-tubulin and visualizing nuclei with DAPI. Five independent replicates were performed, and the total binucleated cells in a population of 200 cells per replicate per group was calculated. The graph shows the mean percentages of binucleated cells ± the SEM (*, $P \leq 0.05$). (d) HCC1954 cells expressing shE2F3 were transfected with either control siRNA or siRNA against *cyclin D1*, and the protein levels of cyclin D1 were assessed by Western blotting. (e) Frequencies of centrosome amplification in the indicated molecular groups were addressed as in panel b.

TABLE 3 Percentage of cells in each cell cycle phase

Cell category	Mean % cells ± SEM[a]		
	G_0/G_1	S	G_2+M
HCC1954 cells			
Proliferating			
HCC1954 pLKO. 1	18.4 ± 0.530	60.6 ± 0.417	21 ± 0.727
HCC1954 shE2F1	29.5 ± 1.192*	56.6 ± 1.096*	13.9 ± 1.070*
HCC1954 shE2F3	30 ± 0.59*	51 ± 0.603*	19 ± 1.071*
0 h after release			
HCC1954 pLKO.1	64 ± 4.67	12.4 ± 1.995	23.5 ± 4.947
HCC1954 shE2F1	63.2 ± 5.151	18.9 ± 1.178*	18.1 ± 4.496
HCC1954 shE2F3	80.7 ± 2.56*	7.2 ± 0.978*	12.1 ± 1.808*
12 h after release			
HCC1954 pLKO.1	72.1 ± 2.611	12 ± 0.46	15.9 ± 2.463
HCC1954 shE2F1	72.9 ± 0.598	18.6 ± 1.341*	8.6 ± 0.843*
HCC1954 shE2F3	84.9 ± 3.008*	9 ± 2.077	6 ± 0.963*
18 h after release			
HCC1954 pLKO.1	35.7 ± 8.413	52.6 ± 7.584	11.7 ± 0.844
HCC1954 shE2F1	34.3 ± 9.225	58.1 ± 6.92	7.6 ± 2.394
HCC1954 shE2F3	34.9 ± 9.658	59.9 ± 8.555	5.1 ± 1.408*
24 h after release			
HCC1954 pLKO.1	15.6 ± 1.476	74.8 ± 1.694	9.6 ± 1.019.
HCC1954 shE2F1	21.3 ± 2.417*	70 ± 2.675	8.7 ± 0.313
HCC1954 shE2F3	13.9 ± 1.458	79.6 ± 1.003*	6.5 ± 1.123*
JIMT1 cells			
Proliferating			
JIMT1 pLKO.1	33 ± 4.723	53.1 ± 2.944	13.9 ± 2.065
JIMT1 shE2F1	28.6 ± 3.266	49.4 ± 3.076	22 ± 2.103*
JIMT1 shE2F3	38.8 ± 3.683	49.6 ± 2.538	11.6 ± 1.453
0 h after release			
JIMT1 pLKO.1	60.7 ± 3.24	25 ± 1.887	14.3 ± 1.541
JIMT1 shE2F1	50.3 ± 1.647*	20.7 ± 1.118*	29 ± 2.235*
JIMT1 shE2F3	59.9 ± 5.962	23 ± 4.995	17.1 ± 1.014
12 h after release			
JIMT1 pLKO.1	71.2 ± 3.023	12.2 ± 1.302	16.7 ± 2.38
JIMT1 shE2F1	60.6 ± 0.604*	8.3 ± 0.727*	31 ± 0.947*
JIMT1 shE2F3	71.8 ± 2.982	12.7 ± 1.439	15.6 ± 1.615
18 h after release			
JIMT1 pLKO.1	53.8 ± 1.536	36.4 ± 2.635	9.8 ± 0.778
JIMT1 shE2F1	41.4 ± 0.107*	40.6 ± 3.062	18 ± 2.043*
JIMT1 shE2F3	50 ± 3.354	39.9 ± 1.55	10.1 ± 0.824
24 h after release			
JIMT1 pLKO.1	39 ± 1.446	51 ± 2.568	10 ± 1.324.
JIMT1 shE2F1	28.2 ± 1.095*	54.2 ± 2.925	17.6 ± 1.89*
JIMT1 shE2F3	40.1 ± 0.61	48.4 ± 1.716	11.4 ± 1.474
SKBR3 cells			
Proliferating			
SKBR3 pLKO.1	37.8 ± 2.175	36.7 ± 1.839	25.5 ± 0.339
SKBR3 shE2F1	49.3 ± 3.089*	32.5 ± 3.636	18.2 ± 2.679*
SKBR3 shE2F3	38.9 ± 4.522	35.1 ± 2.984	26 ± 1.574
0 h after release			
SKBR3 pLKO.1	52.2 ± 2.812	11.9 ± 0.271	35.9 ± 2.96
SKBR3 shE2F1	52.4 ± 4.021	20.3 ± 0.942*	27.3 ± 4.526
SKBR3 shE2F3	45.9 ± 7.804	17.9 ± 3.644	36.2 ± 4.224
12 h after release			
SKBR3 pLKO.1	58 ± 3.416	18.9 ± 0.948	23.1 ± 2.674
SKBR3 shE2F1	62.2 ± 1.392	20 ± 0.889	17.8 ± 2.195
SKBR3 shE2F3	61.6 ± 4.36	14.2 ± 2.323	24.2 ± 2.2
18 h after release			
SKBR3 pLKO.1	53.8 ± 2.239	22.4 ± 1.012	23.8 ± 2.185
SKBR3 shE2F1	60.3 ± 0.611*	20.6 ± 2.565	19.2 ± 2.329
SKBR3 shE2F3	58.4 ± 6.384	15.3 ± 2.118*	26.3 ± 4.27
24 h after release			
SKBR3 pLKO.1	45.8 ± 1.542	32.1 ± 1.669	22.1 ± 3.187
SKBR3 shE2F1	59.1 ± 6.398	22.2 ± 2.887*	18.6 ± 3.999
SKBR3 shE2F3	54.3 ± 7.184	20.9 ± 2.479*	24.8 ± 5.195

[a] *, $P < 0.05$ (as determined by Student t test).

E2F knockdown in HCC1954 cells showed a lower percentage of cells in S phase and in G_2/M phase relative to pLKO.1. No such changes were observed in JIMT1 and SKBR3 cells. Nonetheless, HCC1954 and JIMT1 cells lines reached S phase ~18 h after serum addition, whereas SKBR3 reached S phase 24 h after release. These results show that single E2F activator silencing in Her2$^+$ cells does not greatly affect cell cycle progression.

Nek2 overexpression enhances centrosome amplification in Her2$^+$ breast cancer cells silenced for E2F3. Western blots presented in Fig. 6a showed that decreases in centrosome amplification were associated with the suppression of Nek2 and cyclin D1. Since Nek2 specifically regulates centrosomal functions (78) relative to cyclin D1, which regulates both the cell and centrosome cycles (59, 69, 79), we selected to overexpress Nek2 to establish whether it was sufficient to rescue centrosome amplification in HCC1954 and JIMT1 cells silenced for E2F3. At least two clones of stable cell lines overexpressing GFP-tagged Nek2 were generated: clone 4 was chosen for HCC1954, and clone 1 was chosen for JIMT1, since they overexpressed GFP-Nek2 at similar levels (Fig. 7a). Immunofluorescence microscopy images confirmed GFP-Nek2 expression and localization to centrosomes in interphase (Fig. 7b). Expression of GFP-Nek2 significantly increased centrosome amplification in both HCC1954 and JIMT1 cells downregulated for E2F3 (Fig. 7c). The data show that Nek2 is indeed an important target mediating E2F-dependent centrosome amplification in breast cancer cells.

Downregulation of E2F3 triggers cell death and delays cytokinesis in Her2$^+$ cells. To analyze further the functions of E2Fs in Her2$^+$ breast cancer cells, we investigated the timing of the initiation of mitosis from the previous cytokinesis (visually indicated by the rounding up of cells following interphase) and the timing of the completion of cytokinesis (time from initiation of mitosis to physical separation of cells following cytokinesis) in HCC1954 cells by live cell imaging analysis for 48 h, representing two cell cycles. First, we categorized events into three: divided (successful cell division), not divided (no cell division), and dead cells (Table 4). Our results indicate that fewer shE2F3 and shE2F3; GFP-Nek2 cells underwent cell division compared to the pLKO.1 control. We observed a modest, yet significant decrease in the percentage of shE2F3; GFP-Nek2 cells that did not divide relative to shE2F3 cells. In addition, a significantly higher percentage of shE2F3 and shE2F3; GFP-Nek2 cells were eliminated by cell death before the completion of the experiment compared to pLKO.1 control. Among divided cells, we expanded our analysis into three more subcategories, the first being delayed only in initiation of mitosis. Since most cells start mitosis at approximately 20 to 24 h after the previous cytokinesis, we define delayed initiation of mitosis when it takes more than 28 h to reach mitosis after a previous cytokinesis. Another category was delayed only in cytokinesis (i.e., it takes

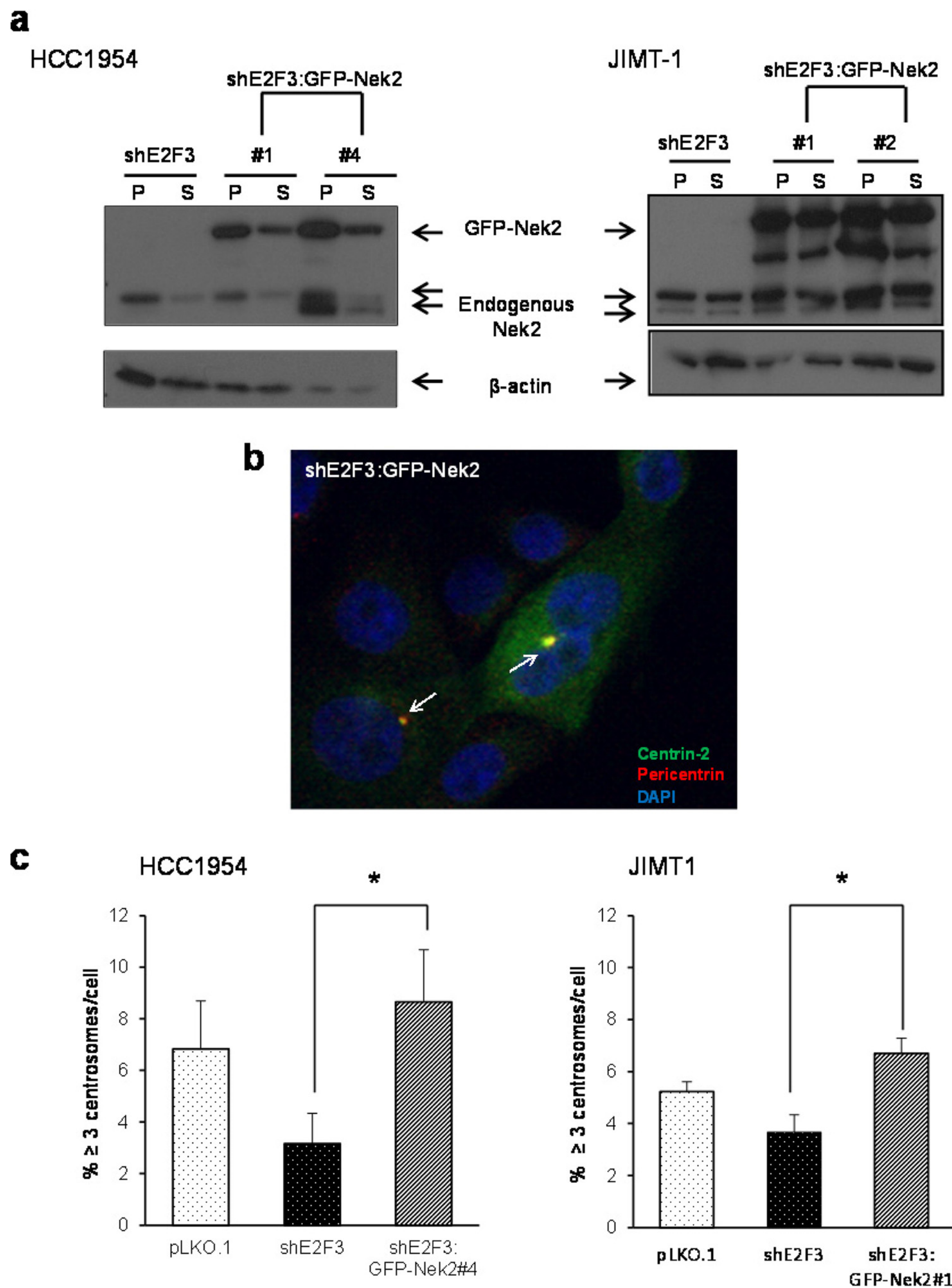


FIG 7 Overexpression of Nek2 in Her2$^+$ cells stably downregulated for E2F3 triggers centrosome amplification. (a) Western blots show shE2F3 HCC1954, and JIMT1 cell populations overexpressing GFP-tagged Nek2 and endogenous Nek2. Western blots were probed with antibodies against Nek2 and β-actin as a loading control. (b) shE2F3 cells overexpressing GFP-Nek2 were visualized by fluorescence microscopy. The centrosomes were detected with antipericentrin antibodies, and nuclei were detected by using DAPI. Arrows indicate colocalization of GFP-Nek2 and pericentrin. Images were obtained under ×40 magnification, cropped, and enlarged for visualization purposes. (c) Centrosome amplification was assayed by pericentrin antibody staining. The assay was repeated three times, and the graph shows the mean percentages ± the SEM (*, $P \leq 0.05$ [comparison of shE2F3 versus shE2F3; GFP-Nek2]).

more than 1 to 2 h from initiation of mitosis to achieve cell division) or cells displaying both delays in initiating mitosis and in completing cytokinesis (Table 4). Overall, we did not detect differences in percentages of cells with delayed initiation of mitosis between controls and shE2F3 cells. In the "delayed only in cytokinesis" and the "delayed in both initiation of mitosis and cytokinesis" categories, the shE2F3 group showed significant increases relative to the controls. On the other hand, GFP-Nek2 overexpression in shE2F3 cells resulted in a timing of completion of cytokinesis similar to that of pLKO.1 cells.

A major controversy in cancer biology is whether binucleated cells can stably undergo mitosis and progress through the cell cycle (46). Thus, we analyzed the fate of binucleated cells (Fig. 8 and Table 4). For this purpose, we included cells already binucleated at 0 h of recording, as well as cells binucleated after cytokinesis. Again, we categorized them into two events: divided and not di-

TABLE 4 Analysis of live cell imaging

Comparison and cell category[a]	Cell line	%	P^b	
			Chi-square test	Fisher exact test
Comparison of the proportion of each event among cell lines				
Divided	pLKO.1	80.39	R	
	shE2F3	59.66	**<0.001**	R
	shE2F3; GFP-Nek2	63	**<0.001**	0.267
Not divided	pLKO.1	9.63	R	
	shE2F3	25.64	**<0.001**	R
	shE2F3; GFP-Nek2	20.08	**<0.001**	**0.033**
Dead	pLKO.1	5.08	R	
	shE2F3	10.6	**<0.001**	R
	shE2F3; GFP-Nek2	12.26	**<0.001**	0.396
Comparison of the proportion of each subevent among divided cells				
Delayed only in initiation of mitosis	pLKO.1	32.68	R	
	shE2F3	37.54	0.151	R
	shE2F3; GFP-Nek2	27.52	0.132	**0.007**
Delayed only in cytokinesis	pLKO.1	6.75	R	
	shE2F3	11.75	**0.014**	R
	shE2F3; GFP-Nek2	6.38	0.838	**0.019**
Delayed both in initiation of mitosis and in cytokinesis	pLKO.1	3.92	R	
	shE2F3	1.43	**0.035**	R
	shE2F3; GFP-Nek2	3.69	0.872	0.065
Comparison of the proportion of subevent of the binucleated cells				
Divided	pLKO.1	65.52	R	
	shE2F3	50	0.254	R
	shE2F3; GFP-Nek2	42.42	0.069	0.571
Binucleated cells divided and died	pLKO.1	31.58	R	
	shE2F3	58.33	0.141	R
	shE2F3; GFP-Nek2	21.43	0.698	0.105
Not divided	pLKO.1	31.03	R	
	shE2F3	25	0.627	R
	shE2F3; GFP-Nek2	30.3	0.950	0.660
Dead	pLKO.1	22.22	R	
	shE2F3	50	0.329	R
	shE2F3; GFP-Nek2	30	1.000	0.607

[a] The percentages of cells whose fate was not determined (mostly because of the time limit) were not incorporated into the table.
[b] The *P* value was calculated using a chi-square test or the Fisher exact test where appropriate. Boldfacing indicates statistical significance. R, reference.

vided. Among divided cells, we further analyzed the portion of binucleated cells that die after mitosis. Under the "not divided" category, we also quantified the portion of binucleated cells dying before reaching mitosis. Although more binucleated cancer cells divided in pLKO.1 control cells (65.52%) compared to shE2F3 (50%) or shE2F3; GFP-Nek2 (42.42%) cells, the results were not statistically significant. Likewise, no significant differences were found in the percentages of binucleated cells dying following mitosis or before reaching mitosis. Overall, these data indicate that E2F3 knockdown compromised survival in the overall population of cells, diminished the fraction of dividing cells, and delayed cytokinesis of HCC1954 cells. On the other hand, Nek2 overexpression in this setting resulted in timing of initiation of mitosis and completion of cytokinesis more closely resembling those of HCC1954 control cells. Also, it is striking to find that a significant fraction of binucleated cells divide, indicating that $Her2^+$ breast cancer cells can tolerate and successfully divide a highly polyploid genome.

DISCUSSION

The frequent deregulation of the E2Fs and the presence of centrosome amplification in the vast majority of breast cancers suggest that they play central roles in breast cancer initiation and/or progression (51–53). Although E2F1 and E2F3 are important mediators of Neu and Myc-initiated mammary tumorigenesis (26, 39), the E2F-dependent activities (including centrosome amplification) contributing to mammary tumors are unclear. Identifying these mechanisms would help to establish the role played by centrosome amplification in breast tumorigenesis. Most progress has been achieved in understanding how centrosomal proteins that include Plk4 and γ-tubulin are regulated by ubiquitination and degradation (80, 81). Another area where major progress has been achieved is that phosphorylation of centrosomal proteins by the G_1-phase Cdks modifies their function, with NPM, CP110, and Mps1 being under such regulation (7, 59, 76, 82, 83). Our laboratory has made major progress in unraveling oncogenic sig-

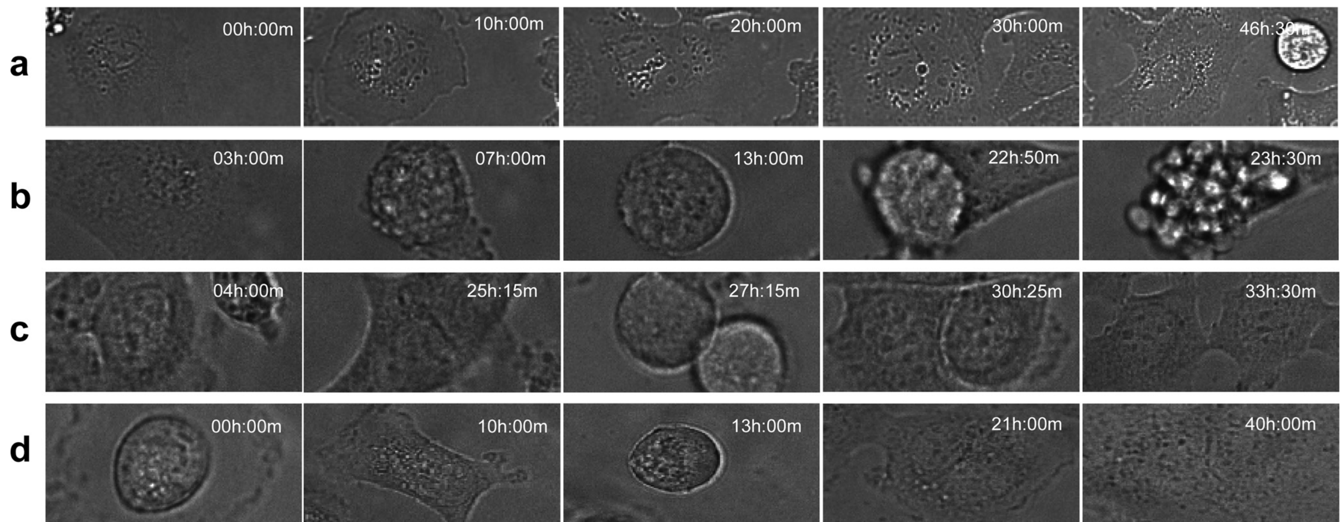


FIG 8 Time-sequential images of the fate of binucleated cells. Several fates of binucleated cells captured at the designated time points are presented. (a) No mitosis; (b) death after mitosis; (c) normal mitosis; (d) generation of binucleated cell after mitosis.

nals and G_1/S regulatory molecules responsible for centrosome amplification in mammary epithelial cells. We showed that centrosome amplification in Her2$^+$ cells and MCF10A cells expressing H-Ras^{G12V} is signaled through cyclin D/Cdk4 and Nek2 (62, 69). While this suggests a role for the E2Fs, the major transcriptional effectors of cyclin D/Cdk4, in centrosome amplification, this remained to be tested. The data shown here, wherein cyclin D1 downregulation does not modify E2F levels or affect the percentages of CA in cells silenced for E2F3, suggest that E2F deregulation in the Her2$^+$ breast cancer cells analyzed here has become independent of cyclin D regulation.

Results presented here indicate that overexpression of E2F1, E2F2, or E2F3a in MCF10A resulted in centrosome amplification and two intermediates to aneuploidy and chromosome instability: binucleation and micronucleus formation. Consistent with this finding, the downregulation of E2F1 or E2F3 significantly diminish centrosome amplification and binucleation in Her2$^+$ cells. Together, these results demonstrate that the E2Fs are sufficient to trigger centrosome amplification and chromosome instability in nontransformed mammary epithelial cells and maintain centrosome amplification and chromosome instability in Her2$^+$ cells. E2F-dependent cancer-driving activities, including hyperproliferation and centrosome amplification, are redundant or unique, dependent on the cellular or tissue context. For example, while the overexpression of E2F1, E2F2, and E2F3 shows redundancy in stimulating the S phase in cultured cells (84–87) and in the context of MMTV-Neu mammary tumors (26, 39), knockout technology indicated the specificity of the functions. For instance, E2F3a/b knockout mouse embryo fibroblasts (MEFs) show cell proliferation, cell cycle, and centrosome cycle defects compared to wild-type, E2F1$^{-/-}$, or E2F2$^{-/-}$ MEFs (7, 88–90). In contrast to experimental mouse models, in our Her2$^+$ breast cancer model, E2F3a or E2F3a/b knockdowns did not affect the S phase, suggesting that this activity is compensated for by the deregulation of E2F1 and E2F2. On the other hand, E2F1 or E2F3 knockdown in Her2$^+$ cells alleviated centrosome amplification, which was not compensated for by the remaining E2F activators.

Our previous study showed that cyclin D1/Cdk4 and Nek2 are required for centrosome amplification in Her2$^+$ cells (62) and in MCF10A cells expressing the H-Ras^{G12V} or H-Ras^{G12V} and c-Myc oncogenes (69), with a positive autoregulatory loop between Cdk4 and Nek2 (62). However, a major unanswered question was whether these targets are regulated via E2F-dependent transcriptional mechanisms. There is evidence that these genes are under E2F control, since E2F sites were reported in the cyclin D1 promoter, and this binding is required to induce cyclin D1 in Her2$^+$ breast cancer cells (91). In addition, Plk4 promoter activity was increased by overexpressing E2Fs in A549 lung carcinoma cells (92), whereas Nek2 is under the repressive control of p107/p130/E2F4 (70). Exploration of the TCGA database showed that overexpression of E2F1, E2F2, and E2F3 in breast cancers strongly associates with the overexpression of the Plk4 and Nek2 transcripts, particularly in ER$^+$ tumors. Expression data in the present manuscript in control cells and cells silenced for E2F1 or E2F3 shows that the E2Fs deregulate various molecules that control the cell/centrosome duplication cycles, including cyclin D1, Plk4, and Nek2, which correlate with centrosome amplification. These data strongly suggest a unique function of E2Fs in centrosome amplification in Her2$^+$ cells by disturbing genes involved in both the cell and centrosome cycles (e.g., cyclin D1) and cells specific to the centrosome cycle (e.g., Nek2 and Plk4).

We demonstrated that Plk4 is under the direct control of the E2F activators in MCF10A and in the Her2$^+$ breast cancer cell line SKBR3. A ChIP assay on putative E2F binding sites on Plk4 promoter in shE2F1 or shE2F3 cells indicated significantly decreased occupancy of E2F1, E2F2, or E2F3 protein binding on these sites compared to pLKO.1 control cells. On the other hand, mutating individual E2F sites in the Plk4 promoter significantly suppressed promoter activity in MCF10A and SKBR3, whereas no significant effects were observed in HCC1954 and JIMT1. However, although the overexpression of E2Fs resulted in elevated Plk4 protein levels, no major changes were observed in two Her2$^+$ cells downregulated for E2F1 or E2F3; a potential explanation is that Plk4 protein levels are under tight ubiquitination and degradation control (93, 94). Another explanation is that given the deep deregulation of E2Fs in Her2$^+$ breast cancer cells, the presence of the remaining

E2Fs occupy the E2F sites in the promoter and increase basal transcription of the reporter promoter. Our data showed that whereas the Nek2 promoter activity is significantly elevated in Her2$^+$ cells relative to MCF10A controls, deletion of the only canonical E2F site in the Nek2 proximal promoter did not affect promoter activity. One potential explanation for this lack of regulation by E2Fs is that another distal E2F site controls Nek2 transcription; however, there are no other E2F consensus sites in >3 kb of Nek2 promoter region. Other potential explanations are that noncanonical E2F sites are required for Nek2 transcription, that E2F regulates another transcription factor directly regulating Nek2 transcription, or that E2F controls other molecules responsible for the stability of the Nek2 transcript/protein levels. Further experimentation is needed to address the source of the E2F-dependent overexpression of Nek2.

Centrosome amplification is caused by various mechanisms such as premature centriole separation/duplication or binucleation (44, 95, 96). Here, we show that there is a close relationship between the E2F activators, centrosome amplification, and binucleation, since the overexpression of E2F1, E2F2, and E2F3a induced these phenotypes, whereas the silencing of E2F1 or E2F3 suppressed them. While we expected that silencing of E2F3 would suppress centrosome amplification by preventing defective cytokinesis in Her2$^+$ cells, live imaging demonstrated that silenced E2F3 results in a significant increase in dead cells and delayed cytokinesis. On the other hand, the overexpression of Nek2 reversed the cytokinesis delays and blocks observed in shE2F3 cells. Our results suggest that silencing of E2F3 restricts unregulated mitosis and cytokinesis in Her2$^+$ cells by restoring checkpoint controls.

We show that Nek2 is a mediator of centrosome amplification in Her2$^+$ breast cancer cells downstream of E2F3. Even though our results indicate that centrosome amplification is a redundant function of the E2F activators, our findings are relevant to the understanding of breast carcinogenesis, since E2F1, E2F3, and Nek2 are frequently overexpressed in breast tumors (26, 97) and negatively impact the outcomes of survival (16, 56, 98). In fact, interference with Nek2 overexpression suppresses tumorigenesis of breast cancer cells (99). However, it is unknown whether the suppression of tumorigenesis relates to centrosome amplification or to the role of Nek2 in regulating various aspects of mitosis (100). Our work has future therapeutic implications, given the published data that deregulation of E2F represents the basis of resistance to various therapeutic agents. Whether the E2Fs signal the resistance via deregulating the cell cycle or through generating genetic diversity through signaling centrosome amplification-aneuploidy remains to be investigated. This research provides data showing that inhibiting the E2F activators and their centrosomal target Nek2 could prevent or reverse centrosome amplification and possibly the occurrence of aneuploidy in Her2$^+$ breast tumors.

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